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Effect of dietary crude protein and forage contents on enteric methane emissions and nitrogen excretion from dairy cows simultaneously

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Abstract. The study aimed to examine, simultaneously, the effects of changing dietary forage and crude protein (CP) contents on enteric methane (CH_4) emissions and nitrogen (N) excretion from lactating dairy cows. Twelve post-peak lactating Holstein cows (157 ± 31 days postpartum; mean ± s.d.) were randomly assigned to four treatments from a 2 × 2 factorial arrangement of two dietary forage levels [37.4% (LF) vs 53.3% (HF) of DM] and two dietary CP levels [15.2% (LP) vs 18.5% (HP) of DM] in a 4 × 4 Latin square design with four 18-day periods. Alfalfa hay was the sole source of dietary forage. Cows were fed *ad libitum* and milked twice daily. During the first 14 days, cows were housed in a free-stall barn, where enteric CH_4 emissions were measured using the GreenFeed system from Days 8 to 14 in each period. Cows were then moved to metabolic cages, where faeces and urine output (kg/cow.day) were measured by total collection from Days 16 to 18 of each period. No dietary forage by CP interactions were detected for DM intake, milk production, enteric CH_4 emissions, or N excretions. There was a tendency for DM intake to increase 0.6 kg/day in cows fed LF ($P = 0.06$). Milk production increased 2.1 kg/day in LF compared with HF ($P < 0.01$). Milk fat content decreased in cows fed LF compared with HF (1.07 vs 1.17 kg/day; $P < 0.01$). Milk contents of true protein, lactose and solid non-fat were greater in cows fed LF ($P < 0.01$). No difference in DM intake, milk yield and milk contents of true protein, lactose and solid non-fat was found between cows fed HP or LP. However, milk fat content increased 0.16 kg/day in cows fed HP ($P < 0.05$). Enteric CH_4 emissions, and CH_4 per unit of DM intake, energy-corrected milk, total digested organic matter and neutral detergent fibre were not affected by dietary CP, but decreased by LF compared with HF ($P < 0.01$). Milk true protein N was not affected by dietary CP content but was higher for LF compared with HF. Dietary N partitioned to milk true protein was greater in cows fed LF compared with HF (29.4% vs 26.7%; $P < 0.01$), also greater in cows fed LP compared with HP (30.8% vs 25.2%; $P < 0.01$). Dietary N partitioned to urinary N excretion was greater in cows fed HP compared with LP (39.5% vs 29.6%; $P < 0.01$) but was not affected by dietary CP content. Dietary N partitioned to faeces was not affected by dietary CP but increased in cows fed LP compared with HP (34.2% vs 27.8%; $P < 0.01$). Total N excretion (urinary plus faecal) as proportion to N intake did not differ between HP and LP, but tended to be lower in cows fed LF compared with the HF diet (64.2% vs 67.9%; $P = 0.09$). Both milk urea N ($P < 0.01$) and blood urea N ($P < 0.01$) declined with decreasing dietary CP or forage contents. Based on purine derivative analysis, there was a tendency for interaction between dietary CP and forage content on microbial protein synthesis ($P < 0.09$). Rumen microbial protein synthesis tended to be lower for high forage and low protein treatments. Increasing dietary forage contents resulted in greater CH_4 emission (g/kg of energy-corrected milk) and manure N excretion (g/kg of energy-corrected milk) intensities of lactating dairy cows. Cows receiving reduced CP diets had low manure N outputs and improved milk true protein production efficiencies, regardless of dietary forage content.

Additional keyword: lactating dairy cow.

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Introduction

Dairy cows convert human inedible substrates into human edible food due to microbial fermentation in the rumen. However, cows also contribute to environmental pollution via methane (CH_4) emissions and nitrogen (N) excretion in manure. Methane is more potent than carbon dioxide (CO_2) because its global warming potential over a 100-year period has been estimated to be 28 times greater (Forster *et al*. 2007). Globally livestock account for 14.5% of total anthropogenic greenhouse gas emissions (FAO 2009). Enteric CH_4 emissions, a product of anaerobic fermentation in the rumen, may account for 25% of total livestock-related greenhouse gas emissions (Steinfeld *et al*. 2006). Methane production is also an energy loss ranging from 2% to 12% of gross energy intake (Johnson and Johnson 1995). Increasing structural carbohydrate contents relative to non-structural carbohydrate contents are associated with greater CH_4

production in the rumen. Therefore, modifications in quantity and quality of dietary forage content can become a promising enteric CH_4 mitigation strategy (Kebreab *et al.* 2006).

Nitrogen excreted into the environment in the form of ammonia, di-nitrogen, nitrous oxide, nitric oxide, and nitrate, have the potential to negatively impact air, soil and water quality (Richardson *et al.* 2009; Menzi *et al.* 2010). This has increased focus on intensive dairy farming systems globally. In addition, only 25% of dietary N intake is retained in milk or meat, with the rest mainly excreted through urine and faeces (Kebreab *et al.* 2002; Calsamiglia *et al.* 2010). Protein consumption over the animal's requirement leads to increased N excretion to the environment (Colmenero and Broderick 2006). The effect of dietary crude protein (CP) content on enteric CH_4 emissions was minimal, however, energy supply (mostly in form of carbohydrates), greatly affected rumen microbial protein synthesis. Firkins and Reynolds (2005) summarised that N excretion is not only related to N intake, but also to other parameters, for example, dietary energy content. Rumen microbial production is maximised with a properly balanced mix of energy and N sources, limiting excess N excretion. The type and amount of carbohydrates in the diet has been shown to affect both N excretion (Castillo *et al.* 2001) and enteric CH_4 emissions (Kebreab *et al.* 2006). Highly fermentable carbohydrates, such as sugars and starch, are more rapidly available compared with other carbohydrate sources, such as cellulose and hemicellulose, in terms of supplying energy to the rumen microorganisms (Stern and Hoover 1979). Moreover, greater amount and concentration of energy in the diet lead to more N partitioning towards milk true protein, and consequently a reduction in faecal and urinary N excretion. Carbohydrates that are ingested can also be used as carbon skeletons for microbial protein synthesis from ammonia.

Several mitigation strategies aiming at reducing enteric CH_4 emissions or N excretions by dairy cows have been studied, albeit independently (Beauchemin *et al.* 2008; Dijkstra *et al.* 2011*a*; Hristov *et al.* 2013). However, nutritional strategies aiming at reducing N excretion may enhance CH_4 emissions and *vice versa* (Bannink *et al.* 2010). More recently, a computer-based analysis has been conducted to address the issue of possible trade-offs between CH_4 emissions and N excretion (Dijkstra *et al.* 2011*a*; Sauvant *et al.* 2014). A mechanistic model simulation attempting to reduce both CH_4 emissions and N excretion simultaneously from cows fed 40 different grass silage-based diets indicated that there may be a trade-off between quantities of N excreted and enteric CH_4 emissions intensity for dietary energy and CP content modifications (Dijkstra *et al.* 2011*a*). The aim of this study was to investigate experimentally, the interaction between dietary CP and energy and quantify their impact on enteric CH_4 emissions and N excretion in lactating dairy cows fed a total mixed ration based on alternative forage sources, such as alfalfa hay.

Materials and methods

Animals and experimental design

All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of California-Davis. The experiment was conducted from July to September of 2014 at the Teaching and Research Facilities of the Department of Animal Science at the University of California-Davis. Twelve Holstein cows (157 ± 31 days postpartum; mean ± s.d.) with an average milk production of 39.3 ± 4.4 kg/day and average bodyweight (BW) of 667 ± 29 kg at the beginning of the study were randomly assigned to four treatments consisting of a 2 × 2 factorial arrangement of two dietary forage levels [37.4% (LF) vs 53.3% (HF) of DM] and two dietary CP levels [15.2% (LP) vs 18.5% (HP) of DM] (Table 1). The experiment had a

Table 1. Ingredient and chemical composition of the experimental diets

	HF		LF	
	HP	LP	HP	LP
Ingredient (% of DM)				
Alfalfa hay[A]	53.3	53.3	37.6	37.2
Steam flaked corn	19.1	27.0	33.7	41.5
Soybean meal	7.5	0.0	12.0	4.3
Cotton seed	5.5	5.5	5.5	5.4
Rolled barley	4.2	4.2	4.1	4.1
Almond hulls	2.6	2.6	2.6	2.6
Dry distillers grains[B]	6.2	5.6	2.4	2.5
Mineral and vitamin mix[C]	1.0	1.0	1.0	1.0
$NaHCO_3$	0.4	0.4	0.4	0.4
$CaCO_3$	0.0	0.1	0.3	0.4
NaCl	0.1	0.1	0.1	0.1
XP-4[D]	0.1	0.3	0.1	0.2
Chemical composition[E] *(% of DM)*				
CP	18.7	15.3	18.4	15.1
NDF	31.0	30.8	24.5	24.3
ADF	24.8	24.6	19.2	19.0
Lignin	6.0	6.0	4.9	5.0
Starch	18.5	24.2	28.7	34.3
EE	3.6	3.8	3.6	3.8
Ash	7.4	7.2	7.0	6.7
P	0.39	0.40	0.40	0.41
Ca	0.83	0.80	0.79	0.77
Na	0.34	0.37	0.35	0.36
K	1.15	1.01	1.07	0.92
Cl	0.36	0.36	0.31	0.29
TDN	68.9	69.1	72.8	73.1
NE_L (Mcal/kg)	1.60	1.60	1.69	1.69
DM (%)	89.4	89.2	88.8	88.6

[A]Contained 91.5% DM and 17.6% CP, 44.2% NDF, 2.5% starch, and 16.3% tdNDF on a DM basis.

[B]Dried distillers grains = dried by-products of whiskey and fuel ethanol production; contained 90.4% DM and 32.2% CP, 28.3% NDF, 6.2% starch on a DM basis.

[C]Mineral and vitamin mix compositions (DM basis): 0.49% CP; 0.185% fat; 0.72% NDF; 11.8% Ca; 5.33% P; 9.16% Na; 0.08% K; 0.005% Cl; 4.27% Mg; 2.11% S; 4466.7 mg/kg of Zn; 208.1 mg/kg of Fe; 2666.7 mg/kg of Mn; 666.7 mg/kg of Cu; 58.7 mg/kg of I; 25.1 mg/kg of Co; 22.7 mg/kg of Se; 0.22% methionine; 0.01% lysine; 533 874 IU/kg of vitamin A (retinyl acetate); 184 800 IU/kg of vitamin D (activated 7-dehydrocholesterol); 4180 IU/kg of vitamin E (dl-α tocopheryl acetate); 58.674 mg/kg of biotin; 933.3 mg/kg of monensin (Elanco, Greenfield, IN, USA).

[D]XP-4 (phosphorus supplement; ICL Performance Products LP, St Louis, MO, USA) contained: 26% of P; 19.3% of Na; 0.03% of S; 30 mg/kg of F; 50 mg/kg of Fe.

[E]$n = 3$.

4 × 4 Latin square design with four 18-day periods. The forage fibre and CP contents in the treatments encompass the ranges in typical lactating dairy cow diets in the USA. For example, surveys by Swanepoel *et al*. (2010) and Silva-del-Rio *et al*. (2010) found forage, acid detergent fibre (ADF), and CP contents of lactating dairy cow diets in California to range between 34–47%, 17–24%, and 16–19% of DM, respectively. Each period consisted of a 7-day adaptation, a 7-day rumen CH_4 emission measurement followed by a 24-h acclimation period to the metabolism cages as described by Knowlton *et al*. (2010) and a 3-day total collection of urine and faeces period. Cows were housed in a single group in a free-stall barn and fed with a Calan Broadbent Feeding System (American Calan, Northwood, NH, USA) during the adaptation period. On Day 15 of each period, BW was recorded after morning milking and cows were moved to an indoor metabolic facility, where they were housed individually in metabolic cages. Each metabolic cage was equipped with a feed trough, a water cup, and a rubber floor. Cows were then fitted with a urinary catheter and acclimatised to the cages until the start of the collection period on the following morning. Four data-logging devices (HOBO UX100–011 Temp/RH 2.5%; Onset Computer Corporation; Bourne, MA, USA) were used to record temperature and humidity during the experiment. The temperature averaged 24.3 ± 0.02°C and humidity 50.8 ± 0.06% during the experiment. A reduction of 9.9% and 5.5% in daily DM intake (DMI) and milk production were detected while cows were housed in the metabolic cages, respectively. Cows were individually fed a total mixed ration prepared once a week because all four treatment diets had low moisture contents (Table 1). Diets were stored within the dairy barn separately and covered with plastic cloth. Cows were fed *ad libitum* twice a day at 105% of previous daily intakes, 60% of which was offered at 0800 hours and the balance was offered at 2000 hours according to Niu *et al*. (2014). Refused feed was removed and weighed before feed delivery in the morning.

Sample collection and analyses

Individual feed ingredients were sampled at each mixing. Representative samples of the ration were sampled on Days 8, 11, 14, and from Days 16 to 18, and orts (12.5%, 1/8 of feed refusals) were sampled from Days 8 to 18 of each period. Feed and orts samples were composited by period. Samples were stored at −20°C until shipped to Cumberland Valley Analytical Services Inc. (Maugansville, MD, USA) for analysis of DM (135°C; AOAC 2000; method 930.15), CP (N × 6.25; AOAC 2000; method 990.03), neutral detergent fibre (NDF) (Van Soest *et al*. 1991), ADF (AOAC 2000; method 973.18), lignin (Goering and Van Soest 1970), starch [(Hall 2008); with correction for free glucose], total ash (535°C; AOAC 2000; method 942.05), and minerals (AOAC 2000; method 985.01).

Cows were milked twice daily at 0600 hours and 1800 hours and milk yield was recorded at each milking using Westfalia milk meters (GEA Farm Technologies, Bonen, Germany) from Days 8 to 14 of each period. From Days 16 to 18, cows were milked using portable milking system (E-Zee Milking Equipment, LLC, Gordonville, PA, USA). Milk was sampled at all milkings from Days 8 to 18 of each period and stored at 4°C with preservative (Bronolab-WII) until analysed for milk fat (Filter B), true protein, lactose and solid non-fat (SNF) by infrared spectroscopy [Fossomatic 4000 Milko-Scan and 400 Fossomatic, Foss Electric, Hillerød, Denmark; AOAC (2000) method 972.160; Central Counties DHIA, Atwater, CA, USA]. Milk urea N (MUN) concentrations were determined using a modified Berthelot procedure (ChemSpec 150 Analyser; Bentley Instruments, Chaska, MN, USA).

Rumen CH_4 emissions were measured from Days 8 to 14 of each period using GreenFeed (C-Lock Technology Inc., Rapid City, SD, USA). GreenFeed is an automated emissions measurement system designed to measure the fluxes (mass per unit time) of gases through the breath of individual cattle that can be detected by CH_4 sensor (Zimmerman *et al*. 2011; Branco *et al*. 2015). In this study, the GreenFeed unit was positioned inside of the free-stall barn for voluntary access, however, GreenFeed was set up so that cows can visit the unit once every 4 h, with daily visits limited to a maximum of 6. Before the study started, cows were trained to use the GreenFeed system for 2 weeks. Gas fluxes through breath were measured during each visit for ~5 min. The GreenFeed system was calibrated three times before and three times after each sample collection period as described by Branco *et al*. (2015). The bait feed was a premix pellet containing (as-fed basis): 56% of alfalfa hay, 10% of soybean meal, and 34% of ground corn. The nutrient composition of bait feed was balanced to be close to the average composition of four treatment diets. Cows received ~220 g of bait pelleted feed of DM during each sampling visit and averaged ~3 sampling visits per day (i.e. maximum of 660 g/cow.day of DM during each sample collection period). The emissions data showed 22.1 ± 1.2 visits per cow during each period with visits occurring throughout different time periods over each day. Methane flux rates (g/h) were first aggregated within 4-h bin periods of a day (0000–0400 hours, 0400–0800 hours, 0800–1200 hours, 1200–1600 hours, 1600–2000 hours and 2000–2400 hours), then daily CH_4 mass for each animal was aggregated through a 7-day rolling average for the daily flux rate (g/day) according to Cottle *et al*. (2015). Ambient air concentration, air flow rate, temperature and humidity were considered and also a CO_2 recovery test was conducted for correcting the measurement of CH_4. The daily actual amount of pelleted feed consumed of each cow was difficult to be precisely measured and is a limitation of the GreenFeed system, hence feed intake was unable to be adjusted for the bait feed intake in order to make CH_4 emission intensity and N excretion intensity comparable.

During the total collection period from Days 16 to 18, faeces were scraped out from the rubber mat immediately after defecation using long handle metal scrapers. Scraped faeces were collected into a plastic container assigned to each cow. Faeces weight was recorded every 2 h from 0900 hours to 2100 hours and every 3 h after 2100 hours. Fresh faecal samples were collected from the rectum six times, twice per day at different times during Days 16 to 18 of each period (0900 hours, 1300 hours, 1700 hours, 2100 hours, 0100 hours, and 0500 hours) for DM and nutrient composition analyses. Approximately 200 g of fresh faecal samples were placed in aluminium trays and oven-dried at 55°C for 72 h. After recording weight, the dried faecal samples of individual cows were stored at −20°C until shipped to Cumberland Valley Analytical Services Inc. Faecal

samples were ground in a Wiley mill (A. H. Thomas Co., Philadelphia, PA, USA) through a 1-mm screen and composited within each period and analysed for the same chemical composition as the feeds. Blood samples were collected from the tail vein at the same time the faecal samples were collected using potassium EDTA vacuum tubes (Greiner Bio-One North America Inc., Monroe, NC, USA). Blood samples were immediately placed on ice, centrifuged at 3000*g* at 4°C for 15 min, and plasma transferred to microfuge tubes, which were stored at −20°C until further analysis. Plasma samples were analysed for blood urea N (BUN) using a Roche Hitachi c501 analyser (6000 series; Roche Diagnostics, Indianapolis, IN, USA).

Urine from individual cow was collected using an indwelling Foley catheter (24 French, 75-cc ballon; C. R. Bard, Covington, GA, USA) connected to 2–3 m of Tygon tubing (Tygon S E-3603 Flexible Tubings; Fisher Scientific, Waltham, MA, USA) running to a 25-L plastic urine collection jar (Nalgene HDPE Jerricans; Fisher Scientific), which was placed in a plastic bucket filled with ~75% ice. Tubes were switched to an empty jar placed in ice at 0900 hours, 1500 hours, 2100 hours, and 0300 hours and urine weights were recorded during four consecutive intervals of 6 h in each collection day. Immediately after recording urine weights, 35 mL of urine was pipetted from each urine jar to 50-mL Falcon tubes (Falcon 50 mL Conical Centrifuge Tubes; Fisher Scientific). Urine samples were acidified with 7 mL 2 M HCl (Knowlton *et al*. 2010) and stored at −4°C until composited at the end of each collection day. The composited samples were stored at −20°C until shipped to Penn State University, State College, PA for further analysis. Diluted urine samples were analysed for allantoin (Chen *et al*. 1992), uric acid (Stanbio Uric Acid Kit 1045; Stanbio Laboratory Inc., San Antonio, TX, USA), and urea N (Stanbio Urea Nitrogen Kit 580; Stanbio Laboratory Inc.). Urine N content was analysed using a Costech ECS 4010 C/N/S elemental analyser (Costech Analytical Technologies Inc., Valencia, CA, USA). Microbial N outflow from the rumen was estimated using urinary purine derivatives (allantoin and uric acid) excretion as described by Hristov *et al*. (2009).

Statistical analyses

Milk production and composition, DMI, nutrient intake and apparent total-tract digestibility, CH_4 emissions, and N excretion were analysed as a replicated design in a linear mixed model using the lmer ('lme4' package) procedure of R statistical language (version 3.2.1; R Foundation, Vienna, Austria). The model was:

$$Y_{ijklm} = \mu + S_i + P_j + C_k(S_i) + F_l + Pr_m + F_l \times Pr_m + e_{ijklm},$$

where Y_{ijklm} is the response variable of interest, μ is the overall mean, S_i is the random effect of sequence (i = 1 to 4), P_j is the fixed effect of period (j = 1 to 4), $C_k(S_i)$ is the random effect of cow nested in sequence (k = 1 to 12), F_l is the fixed effect of dietary forage level (l = LF or HF), Pr_m is the fixed effect of dietary protein level (m = LP or HP), $F_l \times Pr_m$ is the interactions between forage and protein, and e_{ijklm} is the residual error. In all analyses, data points with Studentised residuals outside of ± 3 were considered outliers and were removed from analysis. Rarely more than 1 data point per variable was removed. Statistical differences were declared at $P < 0.05$, and a tendency towards significance was considered at $0.05 < P < 0.10$.

Results and discussion

Because of its potential interactions with dietary nutrient composition (i.e. high energy vs low energy diets), recombinant bovine somatotropin (rBST) injections were stopped 3 weeks before the experiment started. Low forage diets contained relatively low NDF content (24.4% of DM in average) and high starch content (31.5% of DM in average), particularly, the LF and LP diet had a high starch content (34.3% of DM) due to the substitution of alfalfa hay with corn. Hence, the adaptation for the first period was lengthened due to a few cases of acidosis, which was overcome later by adding sodium bicarbonate (0.4% of DM) to the diets. Sodium bicarbonate was added to all the treatment diets throughout the experiment. No significant interaction between dietary forage content and CP content were observed for all the responses tested. Therefore, treatment effects are presented in terms of mean (least-square) response for each forage (HF or LF) and CP (HP or LP) level, and corresponding *P*-values.

Milk production and composition

Impact of dietary forage level (HF or LF) and dietary CP level (HP or LP) on DMI, production traits, and BW of cows during total collection period are presented in Table 2. Although dietary CP content in this study ranged from 15.1% to 18.7%, very few production traits were affected (Table 2). Protein requirements appeared to be met even with the 15.1% CP level basically because the cows were approaching late lactation without rBST injections. Dry matter intake, milk yield, energy-corrected milk (ECM) and feed conversion efficiency (milk yield/DMI) were not significantly affected. Additionally, dietary CP had little effect on yield of milk fat, true protein, lactose, and SNF. In agreement with our study, Leonardi *et al*. (2003) and Colmenero and Broderick (2006) detected little effect of dietary CP content on DMI and milk yield in lactating dairy cows as CP content in the diets increased from 16.1% to 18.9% and 13.5% to 19.4%, respectively. In addition, yield of milk fat, true protein, lactose and SNF were unaffected, in agreement with the findings in Colmenero and Broderick (2006). In contrast, Acharya *et al*. (2015) reported an increase in DMI, milk yield, and yield of milk fat, true protein, and lactose, when dietary CP increased from 14.3% to 16.3% with increasing inclusion rates of canola meal and high-protein dried distillers' grains in the diets. Both Leonardi *et al*. (2003) and the present study found a numerical reduction in milk protein content from 3.25% to 3.18% and 3.11% to 3.10%, respectively, as dietary CP content increased but was insignificant ($P = 0.80$). A similar trend was also shown in NRC (2001) and a review by Ipharraguerre and Clark (2005) showed that the increment in milk yield when cows were fed a higher CP diet was comparatively smaller than those at lower CP diets.

Increasing dietary forage content was associated with a significant drop in milk yield ($P < 0.01$), although the corresponding DMI reduction was not that severe ($P = 0.06$, Table 2). Consequently, feed conversion efficiency (milk yield/DMI) increased for cows fed LF compared with HF diet

Table 2. Effect of dietary forage and crude protein (CP) content on DM intake, milk and milk component yield, milk composition[A] and BW in lactating cows

HF, high forage diet (53.3% forage of DM); LF, low forage diet (37.4% forage of DM); HP, high protein diet (18.5% CP of DM); LP, low protein diet (15.2% CP of DM); SNF, solid non-fat

Item	Forage		Protein			*P*-value		
	HF	LF	HP	LP	SE	Forage	Protein	Forage × Protein
DMI (kg/day)	19.6	20.2	19.9	19.9	0.6	0.06	0.88	0.43
Milk yield (kg/day)	30.2	32.3	31.4	31.2	1.7	<0.01	0.76	0.44
Milk yield/DMI (kg/kg)	1.54	1.60	1.58	1.57	0.07	0.04	0.88	0.96
Milk fat								
%	3.88	3.32	3.68	3.52	0.19	<0.01	0.05	0.41
kg/day	1.17	1.07	1.15	1.10	0.05	<0.01	0.13	0.45
Milk true protein								
%	3.06	3.15	3.10	3.11	0.08	<0.01	0.80	0.43
kg/day	0.91	1.01	0.97	0.95	0.04	<0.01	0.56	0.97
Milk lactose								
%	4.64	4.78	4.70	4.72	0.06	0.02	0.69	0.14
kg/day	1.55	1.68	1.61	1.62	0.10	<0.01	0.81	0.06
Milk SNF								
%	8.74	8.90	8.83	8.81	0.10	0.01	0.81	0.32
kg/day	2.63	2.86	2.76	2.73	0.10	<0.01	0.49	0.90
ECM[B] (kg/day)	31.3	31.3	31.7	30.9	1.3	0.96	0.21	0.78
BW[C] (kg)	653	656	654	655	36	0.45	0.93	0.58

[A]DMI and milk production and composition data were from Days 16 through 18 of each period.
[B]ECM = energy-corrected milk, calculated according to Sjaunja *et al.* (1990).
[C]BW was measured and averaged over Days 8, 11, 14, and 18 of each period.

(P = 0.04). Milk fat yield decreased, whereas other milk component yields increased as forage or NDF content decreased (HF vs LF, $P < 0.05$). In addition, concentration of milk true protein, lactose, and SNF also increased as forage or NDF content declined ($P < 0.05$). Milk fat concentration was 3.32% and 3.88% for cows fed LF and HF, respectively. Therefore, milk fat yield decreased 0.1 kg as dietary forage content increased indicating an increased supply of acetate from rumen fermentation ($P < 0.01$). In agreement with the findings in this trial, Broderick (2003) also reported a reduction of milk yield and milk concentration of true protein, lactose and SNF when dietary NDF content increased from 24.4% to 30.9% of DM. Conversely, milk fat yield was 0.09% lower for the LF diet. Cows fed low NDF and high starch diets appeared to be associated with reduced acetate production rumen as indicated by the declines in milk fat concentration and yield. However, potentially high propionate production typically associated with high starch diets appeared to be responsible for the increasing increments of milk lactose concentration and yield (Bauman and Griinari 2001; Maxin *et al.* 2011). Intake of DM was not significantly affected by the forage content in the diet, consequently, feed efficiency was greater for the LF diet (Broderick 2003). Similarly, Aguerre *et al.* (2011) detected a linear decrease in milk component yield of true protein, lactose and SNF for dietary forage levels ranging from 47% to 68% of DM. However, they also reported numerical declines of DMI and thereby milk yield due to the physical constraint of high forage contents on ruminal digestion (Allen 2000). In this study, alfalfa hay was substituted by soybean meal and steam-flaked corn, which would potentially increase the supply of metabolisable protein through an enhanced rumen microbial protein synthesis (Valadares *et al.* 1999; Broderick 2003). The BW of cows in the present study was not affected by the dietary forage or CP content changes.

Feed intake and apparent total-tract digestibility

Effects of dietary forage and CP contents on nutrient intake and apparent total-tract digestibility during the total collection period are given in Table 3. Intake of NDF and ADF were greater for HF compared with LF ($P < 0.01$), whereas starch intake was greater for LF diets compared with HF diets, as expected. However, forage content in the diets had little effect on intake of organic matter (OM) and CP. However, HP decreased starch intake by 1.2 units compared with LP ($P < 0.01$). As expected, N intake increased 115 g/day in the HP diet compared with LP ($P < 0.01$). Moreover, in agreement with DMI not changing with dietary CP level, a significant effect of dietary CP content was not observed for intake of OM, NDF or ADF. Colmenero and Broderick (2006) found a similar response on CP intake when dietary CP was increased from 13.5% to 19.4% although ADF intake increased with the CP content.

As expected, cows fed LF had higher apparent total-tract digestibility of DM and OM ($P < 0.01$), whereas, the digestibility of NDF, ADF and starch increased as dietary forage content increased ($P < 0.05$). Our findings are in agreement with Broderick (2003) who reported that NDF digestibility to increase with the NDF content in the diet, in which alfalfa silage and corn silage were the main forage sources. Moreover, similar effect of dietary forage content was found for apparent total-tract digestibility of DM and NDF (Neveu *et al.* 2013). The lower total-tract digestibility of NDF for LF diets

could be due to higher passage rates and shorter ruminal turnover times related to the higher DMI (Voelker *et al*. 2002). Dietary CP concentration did not affect apparent total-tract digestibility of NDF and ADF. However, apparent total-tract digestibility of OM, CP, and starch were positively correlated with increasing dietary CP concentration ($P < 0.05$). Moreover, apparent total-tract digestibility of DM tended to increase ($P = 0.07$) as dietary CP level increased. A significant linear increase in apparent total-tract digestibility of N has been previously shown by Broderick (2003) and Colmenero and Broderick (2006).

Methane emission and N excretion

One of the limitations of this study was that only 7 days of adaptation was given for the cows to acclimate for enteric CH_4 emission in each period. Williams *et al*. (2009) reported that more than 4 weeks is needed for methanogens to adapt to dietary changes. However, it has been shown that, rather than the number, it is the distribution of different archaea species that drives the formation of CH_4 in the rumen (Morgavi *et al*. 2010; Abecia *et al*. 2012). The distribution of rumen bacteria and archaea species can be rapidly adapted according to the dynamics of rumen microorganisms determined by gene profiling (Piao *et al*. 2014). In the present study, the enteric CH_4 emission measurements (416 ± 49 g/day; mean ± s.d.) and intensities (19.0 ± 3.4 g/kg of DMI or 12.9 ± 3.1 g/kg of ECM) are comparable to previous studies (Beauchemin *et al*. 2009; Aguerre *et al*. 2011; Moate *et al*. 2011). As expected, dietary forage concentration had a positive effect on enteric CH_4 output (Table 4). Decreasing dietary forage content from 53% to 38%

Table 3. Effect of dietary forage and crude protein (CP) content on daily nutrient intake and apparent total-tract digestibility in lactating cows
HF, high forage diet (53.3% forage of DM); LF, low forage diet (37.4% forage of DM); HP, high protein diet (18.5% CP of DM); LP, low protein diet (15.2% CP of DM)

Item	Forage		Protein			*P*-value		
	HF	LF	HP	LP	SE	Forage	Protein	Forage × Protein
			Intake[A] *(kg/day)*					
Organic matter (OM)	18.3	19.1	18.7	18.7	0.6	0.17	0.97	0.53
Nitrogen (N)	0.54	0.55	0.60	0.49	0.02	0.79	<0.01	0.47
Neutral detergent fibre (NDF)	5.9	4.8	5.4	5.3	0.2	<0.01	0.52	0.66
Acid detergent fibre (ADF)	4.7	3.8	4.3	4.2	0.2	<0.01	0.48	0.91
Starch	4.4	6.7	4.9	6.1	0.2	<0.01	<0.01	0.38
			Apparent total-tract digestibility (%)					
DM	70.3	72.2	71.8	70.8	0.6	<0.01	0.07	0.69
OM	71.2	73.0	72.6	71.5	0.7	<0.01	0.05	0.76
N	69.8	69.1	72.6	66.3	0.8	0.26	<0.01	0.25
NDF	48.1	44.3	46.3	46.1	1.5	0.02	0.88	0.92
ADF	47.3	42.5	45.7	44.1	1.5	<0.01	0.35	0.56
Starch	97.4	96.9	97.4	96.9	0.3	<0.01	0.02	0.41

[A]DMI is shown in Table 2.

Table 4. Effect of dietary forage and crude protein (CP) content on methane (CH_4) emissions[A] in lactating cows
HF, high forage diet (53.3% forage of DM); LF, low forage diet (37.4% forage of DM); HP, high protein diet (18.5% CP of DM); LP, low protein diet (15.2% CP of DM)

Item	Forage		Protein		SE	*P*-value		
	HF	LF	HP	LP		Forage	Protein	Forage × Protein
CH_4 (g/day)	430	399	414	416	12	<0.01	0.82	0.77
CH_4[B] (g/kg of DMI)	20.3	18.0	19.2	19.1	0.9	<0.01	0.78	0.99
CH_4[B] g/kg of ECM	13.5	11.8	12.6	12.7	0.7	<0.01	0.80	0.79
CH_4[C] (g/kg of total digested organic matter)	20.3	18.0	19.2	19.1	0.9	<0.01	0.78	0.99
CH_4[C] (g/kg of total digested neutral detergent fibre)	13.5	12.0	12.9	12.7	0.7	<0.01	0.76	0.87
DMI[D] (kg/day)	21.6	22.6	22.0	22.2	1.1	0.03	0.72	0.62
Milk yield[E] (kg/day)	33.2	35.5	34.0	34.7	2.2	<0.01	0.31	0.31
ECM[F] (kg/day)	32.3	33.9	33.7	32.6	1.7	0.03	0.16	0.27

[A]Rumen methane emissions were measured using GreenFeed (C-Lock Technology Inc., Rapid City, SD, USA). Data were collected and derived from averaged 22.1 ± 1.2 spontaneous measurements over a 7-day period.
[B]Based on milk yield and DMI data during the gas measurement periods.
[C]Total digested OM and NDF were estimated using apparent total-tract digestibility from the total collection period.
[D]DMI = dry matter intake during the gas measurement periods.
[E]Milk yield during the gas measurement periods.
[F]ECM = energy-corrected milk, calculated according to Sjaunja *et al*. (1990).

of DM resulted in a significant 7.2% reduction of enteric CH_4 output. When expressed per unit of DMI or ECM, the reductions were 11.3% and 12.6%, respectively ($P < 0.01$). Moreover, LF diets were associated with 2.3 g and 1.5 g less CH_4 emissions per kg of total digested OM and NDF, respectively ($P < 0.01$). The results were in agreement with Aguerre *et al*. (2011) who reported a linear reduction in CH_4 emissions in cows receiving alfalfa silage and corn silage-based diet with forage contents ranging from 47% to 68% of DM. The lower CH_4 emissions of LF diets compared with the HF diets could be due to a shift in nutrient digestion from structural (fibrous) to non-structural carbohydrate (starch and sugars) fermentation favouring propionate formation and resulting in lower acetate : propionate ratio in the rumen (Johnson and Johnson 1995; Bannink *et al*. 2008). Moreover, the reduction of rumen pH in cows fed LF diets would also decrease rumen CH_4 emissions through inhibition of methanogenesis (van Kessel and Russell 1996). Reduced CH_4 emission intensity to decreasing dietary forage content was reported in other species, such as sheep (Waghorn *et al*. 2002) and beef cattle (Lovett *et al*. 2003). Cows fed LF may have a more rapid digesta passage rate compared with HF, which would reduce fibre digestibility in the rumen. In addition, the reduced NDF total-tract digestibility of LF (44.3% vs 48.1%, $P = 0.02$) also suggests the possibility of less acetate in the rumen and therefore less hydrogen substrate for CH_4 formation. Dietary CP content is generally regarded as a negligible factor on CH_4 emission, consistently, dietary CP levels did not affect either the absolute amount of CH_4 emitted or CH_4 emission intensities in the present study.

Effects of the dietary forage and CP levels on N partitioning during the total collection period are presented in Table 4. Urine volume, urinary N and urea N output, and total N (urinary plus faecal) output increased as dietary CP increased ($P < 0.01$). As N intake increased substantially (601 vs 486 g/day), milk N efficiency (milk true protein N/N intake) declined from 30.8% to 25.2% (Table 5). Consistently, Hristov *et al*. (2004) reported that N efficiency reduced 2.7% when CP increased from 15.8% to 18.3%. In line with the increased urea N output in the present study, urine output was also considerably increased as dietary CP level was elevated (26.6 vs 21.6 kg, $P < 0.01$). Urinary urea primarily contributes to urine osmolality, which in turn determines total urine mass (Appuhamy *et al*. 2014). In agreement with Castillo *et al*. (2001), the present study also did not find differences in faecal N output (g/day) between cows fed HP and LP diets. However, the reduction of N total-tract digestibility resulted in more N going to faeces as N intake was less among cows receiving LP. Similarly, dietary CP content positively affected N excretion with the majority excreted in urine, in lambs (Bunting *et al*. 1987) and beef cattle (Koenig and Beauchemin 2013). As expected, both BUN and MUN concentrations of HP were greater compared with LP ($P < 0.01$).

Diets with low fibre content were associated with increased milk true protein N output (158 vs 143 g/day, $P < 0.01$). Consequently, milk N efficiency was higher for LF diet compared with HF (29.4% vs 26.7%, $P < 0.01$). Hence, a greater proportion of digested N appeared to be incorporated into milk protein in cows fed low forage diets, compared with those fed high forage diets. Consequently, a smaller proportion

Table 5. Effect of dietary forage and crude protein (CP) content on urinary purine derivatives (PD) and nitrogen (N) excretion and secretion in lactating cows

HF, high forage diet (53.3% forage of DM); LF, low forage diet (37.4% forage of DM); HP, high protein diet (18.5% CP of DM); LP, low protein diet (15.2% CP of DM); BUN, blood urea nitrogen; s.e., standard error

	Forage		Protein			*P*-value		
Item	HF	LF	HP	LP	s.e.	Forage	Protein	Forage × Protein
N intake (g/day)	543	545	601	486	19	0.79	<0.01	0.47
Urinary and faecal excretions								
Urine output (kg/day)	25.3	22.9	26.6	21.6	1.4	<0.01	<0.01	0.26
Urine N (g/day)	204	181	237	149	12	0.04	<0.01	0.52
% of N intake	36.1	33.0	39.5	29.6	1.4	0.11	<0.01	0.67
Urea N (g/day)	141	121	165	96	3.0	<0.01	<0.01	0.61
Faecal output (kg/day)	35.8	33.5	34.6	34.7	1.4	<0.01	0.95	0.85
Faecal N (g/day)	164	169	167	166	7	0.19	0.92	0.55
% of N intake	30.6	31.3	27.8	34.2	0.8	0.31	<0.01	0.23
Urinary and faecal N excretion (g/day)	370	349	403	315	16	0.09	<0.01	0.50
% of N intake	67.9	64.2	67.4	64.8	1.5	0.09	0.22	0.72
Milk true protein N (g/day)	143	158	151	150	6	<0.01	0.56	0.97
% of N intake	26.7	29.4	25.2	30.8	0.8	<0.01	<0.01	0.92
Urinary PD excretion (mmol/day)								
Allantoin	462	494	502	453	27	0.42	0.22	0.09
Uric acid	16.3	15.3	17.1	14.5	0.8	0.09	<0.01	0.62
Total PD	478	509	519	468	27	0.44	0.20	0.09
Microbial N synthesis in the rumen[A] (g/day)	317	340	348	310	20	0.43	0.20	0.09
MUN (mg/dL)	14.9	13.8	17.0	11.7	0.6	<0.01	<0.01	0.67
BUN (mg/dL)	15.4	13.0	17.1	11.3	0.5	<0.01	<0.01	0.56

[A]Microbial N synthesis in the rumen were estimated based on urinary PD excretion as described in Materials and methods.

of digested N appeared to be catabolised by splanchnic tissues as indicated by the lower MUN and BUN concentrations of LF diets (Table 5). Moreover, in line with MUN and BUN changes, less urea N was excreted in urine from cows receiving the low fibre diet ($P < 0.05$). There was a tendency for a dietary forage by dietary CP interaction on urinary allantoin and total purine derivative excretion, which was also reflected by the estimated microbial N synthesis in the rumen ($P = 0.09$). Cows receiving HF were associated with greater microbial protein synthesis when dietary CP level was low (322.3 vs 309.9 g/day, data not shown), whereas cows receiving LF were associated with greater microbial protein synthesis when dietary CP level was high (382.9 vs 297.4 g/day, data not shown). This could be because of synchronisation of energy supply and microbial protein synthesis in the rumen indicating the importance of providing adequate energy, especially rapid fermentable carbohydrates for maximising rumen microbial protein synthesis in order to reduce N excreted through urine and faeces.

One of the strategies to mitigate CH_4 emission is to reduce structural carbohydrate content in the diet (Hristov *et al*. 2013). Such strategy also appeared to reduce N excretion of cows fed adequate amounts of carbohydrate and N (Bach *et al*. 2005). However, interaction of dietary CP and forage was not detected in our study, which might be due to the excess CP intake in cows fed both LP and HP diets. This agrees with the common understanding that avoiding overfeeding dietary N is the number one N excretion mitigation strategy. Our study showed that decreased dietary CP content reduced N excretion but CH_4 emission was not affected. Similarly, enteric CH_4 emissions decreased in cows fed low forage regardless of the CP content. However, the reduction of diet forage content needs to be carefully managed as a mitigation strategy of CH_4 because of acidosis-related concerns. Moreover, we did not observe a trade-off between the dietary modifications aiming at mitigating enteric CH_4 emissions and N excretions in the present study. This does not agree with the mathematical simulations by Dijkstra *et al*. (2011*b*) who showed an existence of such a trade-off. This discrepancy could be (1) the range of diets was greater in the simulation study and (2) forage source was different, i.e. grass silage versus alfalfa hay-based diet in simulation and the present study, respectively. The simulation contained four types of grass silage with five different supplementations using two levels of concentrate (20% or 40% of DM; Reijs *et al*. 2007; Dijkstra *et al*. 2011*b*). Even with the wide range of diets, the negative correlation in the simulation was not strong ($r^2 = 0.22$, Dijkstra *et al*. 2011*a*). Thus, further studies are needed to better understand the relationship between N excretion and CH_4 emissions using more diverse diets that are based on different forage sources to come up with strategies that jointly minimise CH_4 emissions and N excretion.

Conclusions

No interactions between dietary CP and forage contents were found in the present study. Reducing forage content in the diet resulted in lower NDF digestibility, higher ECM, lower CH_4 emissions (g/day), as well as lower urine N excretion regardless of the dietary CP content. Decreasing dietary CP content reduced manure N output (g/day) and increased partitioning of N to milk protein, but no effect on CH_4 emissions was observed. Reducing forage content reduced enteric CH_4 emission intensity. Moreover, reducing forage content also increased N partitioning to milk and tended to reduce manure N output, independent of dietary CP content. Methane emissions and N excretions in lactating dairy cows can be independently reduced by reducing dietary forage and dietary CP contents, respectively.

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